PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [4]: C12N 9/96, G01N 33/531, 33/543 G01N 33/52, A61K 39/00 — A1

(11) International Publication Number: WO 87/ 00196

(43) International Publication Date: 15 January 1987 (15.01.87)

(21) International Application Number: PCT/GB86/00396

(22) International Filing Date: 9 July 1986 (09.07.86)

(31) Priority Application Numbers: 8517352 8613066

(32) Priority Dates: 9 July 1985 (09.07.85) 29 May 1986 (29.05.86)

(33) Priority Country: GB

(71) Applicant *(for all designated States except US)*: QUADRANT BIORESOURCES LIMITED [GB/GB]; Manor Farm, Soulbury, Near Leighton Buzzard, Bedfordshire (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : ROSER, Bruce, Joseph [AU/GB]; The Old Vicarage, Church Lane, Balsham, Cambridgeshire (GB).

(74) Agent: ABLEWHITE, Alan, James; Marks & Clerk, 57/60 Lincolns Inn Fields, London WC2A 3LS (GB).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB, GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

Equivalent to EP 0229810

(54) Title: PROTECTION OF PROTEINS AND THE LIKE

(57) Abstract

Sensitive proteins and other macromolecules, such as enzymes, antibodies, antigens, serum complement, fluorescent proteins, vaccine components, polysaccharides such as agarose etc, can be preserved by drying at ambient temperature and at atmospheric pressure in the presence of trehalose. A porous matrix impregnated with trehalose is provided as a receiver for a blood or other liquid sample to be dried.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GA	Gabon	MR	Mauritania
AU	Australia	GB	United Kingdom	MW	Malawi
BB	Barbados	HU	Hungary	NL	Netherlands
BE	Belgium	IT	Italy	NO	Norway
BG	Bulgaria	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
DE	Germany, Federal Republic of	LU	Luxembourg	TG	Togo
DK	Denmark	MC	Monaco	US	United States of America
FI	Finland	MG	Madagascar		
FR	France	ML	Mali		

Protection of Proteins and the like

This invention relates to the protection of proteins and other macromolecules against denaturing during drying.

Macromolecular compounds, especially proteins and polypeptide-containing compounds, commonly exist in their naturally occurring hydrated state in the form of complex, three-dimentional folded conformations generally known as tertiary structures. Very frequently, the activity of the compound, whether as an enzyme, antibody, antigen, flavourant, fluorescent, gelling agent etc., is critically dependent on the tertiary structure and is severely reduced or even eliminated if the structure is disturbed, even though the chemical empirical formula of the compound may not have changed. This is a very serious problem when the protein etc is required in a dry state, for storage etc.

In order to combat this problem various solutions have been proposed. Enzymes for dry immunoassay kits have been protected in liposomes. Vaccines cannot easily be stored in the freeze-dried state and so have to be stored in bulky ampoules. Fluorescent proteins

such as the phycobiliproteins of use in immunoassay techniques lose their ability to fluoresce if dried and so cannot be used in dry kits. Yet a further example is the loss of antigen-binding capacity of monoclonal antibodies bound to a resin substrate and dried.

There is thus an urgent need for a means of protecting such substances from deactivation on drying. We have now found that this object can be achieved by drying the compound in the presence of trehalose.

Trehalose, α-D-glucopyranosyl-α-D-glucopyranoside, is a naturally occurring non-reducing disaccharide which has previously been associated with cell protection. It is known that some organisms, both plant and animal, can resist desiccation to very low levels of body water during drought conditions. These organisms include brine shrimps cysts (Artemia salina), the resurrection plant (Selaginella lepidophylla) and bakers yeast (Saccharomyces cerevisiae). They all share, as a common feature, the presence of large amounts of trehalose in their cells.

A body of work exists on the effects of various carbohydrates including trehalose on the stabilisation of cell membranes during freezing and dehydration. This work shows trehalose to be significantly superior to other carbohydrates in protecting cellular organelles

from the deleterious effects of the loss of bound water.

(Crowe, J.H., Crowe L.M. and Mouradian R. (1983) Cryobiology 20, 346,356;

Crowe, J.H., Crowe L.M and Chapman D. (1984) Archives Biochem. Biophysics 232, 400-497; and

Crowe L.M., Mouradian R, Crowe J.H., Jackson S.A and Womersley C. (1984). Biochimia & Biophysica Acta 769, 141-150.)

While there is no consensus view as to how trehalose exerts its protective effects on cells, one hypothesis is that it substitutes for the bound water on membrane components of the living organism and prevents denaturation due to loss of bound (structural) water. We have now found that the effect is exhibited not only in living cells, but surprisingly also in macromolecules themselves in a purified, isolated state.

EP140489A of Wako Pure Chemical Industries discloses an immunoactive substance (e.g. antibody) on a carrier, e.g. glass beads, stabilized against drying at ambient temperature by immersing in a solution of a sugar, optionally together with a protein such as bovine serum albumin. A considerable number of sugars are mentioned as being of use (ribose, glucose, fructose, mannose,

galactose, maltose, lactose, sucrose, oligosaccharides and polysaccharides); preferred are lactose, sucrose or dextrin solutions. There is no mention, however, of trehalose.

Patent Application GB 2009198(A) (Behringwerk AG) discloses the lyophilisation of meningococcal polysaccharide and trehalose; British Patent Application GB 2126588A (Asahi Kasei KKK) discloses the stabilization of tumor necrosis factor (TNF) to lyophilisation and freezing by including either a non-ionic surfactant or trehalose (or another sugar); and Published Japanese Patent Application J 58074696A (Iatron Laboratories) discloses the freeze drying (lyophilisation) of ATP in the presence of trehalose.

It will be noted that these three publications disclose that trehalose will stabilize delicate materials while they are freeze dried. Freeze drying (lyophilisation), as a technique, was devised as being the only way certain sensitive materials can be handled. Ordinary drying at ambient or elevated temperature and at atmospheric or reduced pressure causes irreversible degradation of very many such substances, so much so that it has always gone without saying in the biological field that sensitive proteins etc cannot and must not be dried at ambient temperature. In freeze-drying, the water is removed

under high vacuum from the solid material. In this way, the problems of liquid film denaturation of proteins, thermal instability etc are avoided. It has not previously been realised that trehalose can not only provide total protection during drying at ambient temperatures as high as 37° or 40°, but also allow intramolecular electronic processes such as fluorescence and photoconductivity to occur in the dry state.

According to this invention we provide a method of protecting proteins and other macromolecules against denaturation during drying, comprising subjecting an aqueous system containing the protein or the like to drying in the presence of trehalose. The trehalose is conveniently dissolved in the aqueous system and may be present in any effective amount. In general a concentration of from 0.05 to 20% by weight of trehalose is desirable, especially about 0.1 to 10%, e.g. about 2.5% by weight.

The drying process may be achieved by simple air drying of aqueous systems containing the protein and trehalose on a suitable surface, e.g a glass or plastic plate, or dish, a film of plastic or nitrocellulose or a porous support such as a paper. The drying is preferably at atmospheric pressure.

The presence of the trehalose in the dried product

has no effect on the physiochemical or biological properties of the macromolecular substance although there will obviously be rare situations (for example protection of a trehalose-degrading enzyme) where problems will arise. In the case of other enzymes antibodies, antigens and fluorescent markers no problems arise and the compound is as active as if it had been stored in the hydrated state.

Many enzymes are now used in various test procedures, or are themselves tested for in various assays. According to the present invention the enzyme or the substance containing an enzyme can be dried and stored and can be reactivated when required by the addition of wateror a buffer solution. For example, peroxidase (e.g. from horseradish) and alkaline phosphatase (mammalian) can be preserved in this way. The complex "cascade" of emzymes found in serum, which is activated by the antibody-antigen combination in an immune response (P.J.Lachman and M.J.Hobart, "Handbook of Experimental Immunology" ed. D.M.Weir, Blackwell 1978) is notoriously labile. Serum itself contains about 70-75 mg/ml of proteins of which only about 2 mg/ml comprise the complement fraction. Serum containing complement (or a purified complement fraction derived from it) cannot be dried, it has to be stored under very low temperature conditions (-70° to 196°C). It can be lyophylised, but even this technique destroys

some of the activity. Thus, for example, lyophylised complement cannot be used in lymphocyte cytotoxicity assays. Most surprisingly, we find that complement activity can be completely preserved according to the present invention by drying in air at room temperature. The dried material can be stored at, say, 37°C for weeks and can be reconstituted with its activity retained.

We find that the amount of trehalose required to be present in the serum sample is generally proportional to the content of protein. For example a 50% by weight serum dilution in water requires at least 0.22M trehalose (i.e. about 75g/l) while a 25% dilution requires at least 0.15M trehalose (i.e. about 51g/l) for complement protection. Other serum components e.g. serum antibodies need rather less. In general, therefore, a weight ratio of trehalose to protein (or other macromolecule) of at least 1.4:1, preferably at least 2:1 and most preferably at least 3:1 is suitable, e.g. up to 10:1.

The preservation of serum and serum complement, antibodies and antigens, means that according to the present invention it is also possible to preserve many types of vaccine by drying in the presence of trehalose. Antisera are used to treat various toxic conditions and immunising vaccines containing killed microorganisms are widely used. Both types of vaccine

require storage under cool, hydrated conditions, or under deep freeze conditions. Dried vaccines are now possible by the technique of this invention.

Another problem of storage and preservation occurs in the field of agarose gels. For many biochemical techniques agarose gels are used as substrate, e.g. electrophoresis, Ouchterlony diffusion, immuno-electrophoresis etc. Gels of 1-3% are widely used as separation media in these techniques. At the present time they have to be made fresh in the laboratory for each experiment by heating solid agarose in buffer until it dissolves, then pouring the molten agarose into a former and cooling to gel the agarose. Pre-formed gels can only be stored for relatively brief periods in humidified containers. If allowed to dry, the gels undergo irreversible collapse of gel structure and cannot be reconstituted by simple rehydration in water or buffer.

According to the present invention 2 and 3% agarose gels containing 2-20% trehalose can be dried and subsequently rehydrated. We have compared their rehydration characteristics with gels dried without trehalose we have found that the dried gels can be completely rehydrated to give gels of the original depth and water content. This means that dried agarose gel plates can be kept indefinitely without the need for

special containers and can simply be rehydrated when required for use.

According to the invention it is also possible to preserve by drying at ambient temperatures fixed whole red blood cells and especially fixed whole red blood cells to which are attached labile molecules such as antibodies, and antigens, e.g. for use in the extremely sensitive form of immunoassay known as the haemagglutination assay. In this, either antigen or antibody is coupled chemically to the surface membrane of erythrocytes and the appropriate ligand can be detected by its ability to cause the red cells to clump together into a visible aggregation pattern. Where antigen is coupled to the red cell, the potency of an antibody can be assessed by titrating the antibody out in dilution steps and observing the point at which it no longer causes agglutination/aggregation of the red blood cells. Where antibody is coupled to the red cell, the precise amount of antigen in an unknown solution e.g. blood can be estimated by detecting the titration end point as above. For this to work, the antigen must carry more than one antigen site per molecule capable of being "seen" by the antibody on the red cells.

This assay requires a lot of preparation. The red cells are usually used fresh and need to be washed, chemically coupled with either antigen or antibody and

then added to the titrated dilution of ligand.

We have found that we can couple antigens and antibodies to red cells which are fixed with a histological fixative such as glutaraldehyde and then dry the coupled red cells in the presence of trehalose. Upon rehydration the coupled, fixed red cells can be resuspended to form a perfect single cell suspension which then behaves as a fresh red cell suspension does in haemagglutination assays. In this use the trehalose serves two functions:

1. It completely preserves the function of the antibody or antigen coupled to the fixed red cell membrane.

2. It completely preserves the fixed red cells themselves so that they completely resuspend in buffer to give a single cell suspension. When fixed red cells are dried in the absence of trehalose they undergo irreversible spontaneous agglutination and cannot be used.

Thus an instant haemagglutination assay kit can be constructed in the dry state which works in an entirely conventional way when the antigen or antibody which is to be measured is simply added to the dried red cells in buffer.

R Phycoerythrin is a phycobiliprotein which can be easily purified from the marine rhodophyte, Rhodymenia. It is brilliantly fluorescent in the red-orange with an emission maximum of 575 nm. Its bright fluorescence and high quantum yield are dependent upon the spatial distribution and configuration of the phycourobilin and phycoerythrobilin chromophores in the molecule. This fluorescent protein of choice for many fluorescent assays because it is 20 times brighter than fluorescein on a molar basis and can easily be coupled to probes such as antibody or Avidin/Streptavidin for use in fluorescent antibody or DNA/RNA hybridisation assays.

A serious drawback to the use of this reagent is its succeptibility to fluorescence fading in aqueous solution. This gives the fluorescence a very short half life. It is though to be due to damage to the phycoerythrin molecule due to the creation of free radicals in the aqueous solvent when illuminated with the exciting wavelength for fluorescence. Drying the molecule prevents creation of free radicals and therefore the fading but destroys more than 90% of the fluorescence because the three dimensional structure of this large protein (240 Kd), upon which its fluorescent properties depend, collapses when dried.

We have found that this molecule can be dried completely in the presence of trehalose without loss of

its fluorescent properties. This means that this protein can now be used in assays which require prolonged or repeated exposure to high intensities of exciting light without fading and with complete retention of its intense fluorescence. Fading can be reestablished by washing away the trehalose and irradiating the molecule in an aqueous solvent.

A major requirement for the preservation of protein structure and function is in blood samples taken for clinical measurement of levels of antibodies, antigens and enzymes. At present these samples are usually stored for short periods of 4° or for prolonged periods at -20° or lower in a deep freeze. They cannot be dried.

An attractive alternative would be to store serum or blood samples in the dry form where the structure and function of the blood components were protected by drying in the presence of trehalose. A simple and elegant method of achieving this is to use, as the sample storage medium, a porous matrix previously impregnated with trehalose, on to which the blood or serum sample is applied and which is allowed to dry again. The drying can be effected at any convenient ambient temperature, e.g. those encountered in tropical areas. The content of the trehalose in the matrix is conveniently from 5 to 25% by weight of dry matrix, e.g. 7.5 to 20%. The matrix may comprise any suitable inert

material, e.g. cellulose or glass-based papers, porous plastic films, fabrics etc. For ease of use, cellulose filter papers are preferred. For ease of retrieval of the absorbed protein, the matrix is conveniently a thin sheet or web.

While we do not wish to be bound by theory, it seems likely that the unique properties of trehalose in preserving the structure and function of proteins and other macromolecules in the dry state is due to hydrogen bonding of trehalose molecules via their hydroxyl groups to appropriate groups on the macromolecule. In this way trehalose takes the place of structural (bound) water molecules so that there is no collapse of macromolecular structure upon drying. The trehalose acts as a dry scaffold maintaining the structural integrity of the macromolecule.

Many macromolecular functions require mobility of the macromolecule e.g. antibodies have to physically move into close proximity to their antigen in order for binding to occur through electrostatic and hydrophobic interactions. The same is true of enzymes binding their substrates. Minor molecular motion is also required for other macromolecular functions. Thus haemoglobin undergoes rotation and movement of certain amino acids during the reversible binding of oxygen and carbon dioxide. None of this molecular mobility is observed in

the dry state. Thus we have dried haemoglobin in the oxy and carboxy forms, and these molecules retain their spectral properties when dried in the presence of trehalose.

Exposure of carboxy haemoglobin, dried in trehalose, to 100% oxygen does not result in conversion to the oxy form whereas this occurs within a few minutes in aqueous solution. In contrast, the fluorescence of R-phycoerythrin involves "movement" only on the sub atomic level. Absorption of light energy at low wavelengths e.g. (488 nm) causes electrons in the chromophores to shift to higher energy orbits. Fluorescence at longer wavelengths is due to the emission of photons when these electrons jump back to lower energy orbits. The preservation of the fluorescence of R-phycoerythrin in the dried state by trehalose thus indicates that electronic properties of macromolecules can be completely preserved by this technique. This method is therefore of great interest in the application of biological (and synthetic) macromolecules in new electronic applications. Examples include proton and electron pumping (such as is mediated, on exposure to light, by bacteriorhodopsin and rhodopsin). The ability to dry proteins and other macromolecules and preserve their three dimensional shape and functions offers the possibility of molecular electronics including direct conversion of solar energy

into hydrogen or electric current using biological molecules which have an inherently high efficiency and the construction of sensors and electronic circuits (including computers) on the molecular size scale.

The following Examples illustrate the invention further.

Example 1

The phycobiliprotein R-phycoerythrin was purified from a marine Rhodophyte. This brilliantly fluorescent red protein was blotted onto nitrocellulose membrane or glass fibre filter paper in phosphate buffer with and without 10% w/v added trehalose and dried at room temperature. In the absence of trehalose the red protein turned purple and lost its ability to fluoresce orange/red when illuminated with blue light. In the presence of 10% trehalose the protein retained its colour, and fluoresced just as brightly as the protein in solution, even though it was completely dry.

We have also completely preserved the fluorescence of phycoerythrin coupled to antibodies which have bound to the surface of lymphocytes, by drying them in the presence of trehalose.

Example 2

We have also studied the ability to trehalose to preserve the antigen-binding capacity of antibodies when dried. This can best be illustrated by its application to a very sensitive two site Enzyme Linked Immunoassay (ELISA) for Class I transplantation antigens.

In this assay a monoclonal antibody YR5/310 was bound to polystyrene surfaces in a 96 well microtitre plate (Nuc Immunoplate) by overnight incubation. We then dried 24 wells of the plate in the absence of trehalose, dried 24 wells in the presence of 10% added trehalose and left 48 wells wet in phosphate buffer. The drying was at 37° overnight.

We then added serum containing the relevant class I molecules and incubated at room temperature for 5 hr. The plates were washed and then a second antibody, YR5/12, labelled with biotin was added for 1 hr. The plates were washed again and incubated with the substrate 3,3',5,5'-tetramethyl benzidine for 20 min. The reaction product was then measured in an automatic ELISA plate reader. The results (Fig.1) show that drying the bound antibody in the absence of trehalose causes a loss of >90% of the binding activity while drying in the presence of added trehalose preserves the antibody activity completely so that it gives ELISA

results comparable to those given by non dried antibodies.

Example 3

Dry plate blood typing assay

A four well tissue culture dish (Nunclon 3/132 multidish 4,Nunc Denmark) is used. Other cheaper alternatives could be used, e.g. "blister packs" made from thin PVC sheet. Also Standard microtitre plates of 94, 48 or 24 wells could also be used.

Manufacture

Three wells contain antibody and one (well 4) contains only 50µl of monoclonal mouse IgM antibody to the A blood group substance (64 i.u. per ml - from the Blood Group Reference Laboratory, Radcliffe Infirmary, Oxford.)

Well 2 contains 50µl of monoclonal mouse IgM antibody to the B blood group substance (64 i.u. per ml - same source as above).

Well 3 contains 50µl of monoclonal human IgM antibody to the Rh D antigen (1 mg per ml, produced in our laboratory).

All wells also contain 50µl of 0.1% to 10% w/v trehalose solution in distilled water plus 5 units of heparin and 0.01% sodium azide. The plates are dried overnight at 37°C in a warm room. They can then be stored at room temperature indefinitely.

Use

Add 2 drops (approx 100µl) of water (distilled or tap water) to each well and spread by rocking plate for 2-5 sec. Add 1 drop of whole blood and rock gently to mix blood and well contents. Rock gently every few seconds for 5 min.

Results

Blood containing red cells which are positive for the A, B or Rh antigen will give a clear, coarse macroscopic agglutination pattern within 30 secs in the appropriate well(s). O, Rh-ve blood gives no agglutination in any well.

Advantages

1. The plates are indefinitely stable at room temperature. They have been stored at 37° for 2 months without loss of activity.

2. The test is very convenient and fast. No equipment is required. Whole blood is used. The erythrocytes do not need to be separated or washed. A finger prick will give enough blood for ABO and Rh testing.

3. Trehalose preserves 100% of the antibody activity. A test of five sugars all used at 5% final concentration in a titrated haemagglutination assay gave the following results.

	% activity retained in HA titre
Wet antibody (a human IgM anti-D)	100
Dried Ab + no additive	3.7
Dried Ab + trehalose	100
Dried Ab + maltose	11.1
Dried Ab + sucrose	11.1
Dried Ab + raffinose	3.7
Dried Ab + glucose	1.2

An additional titration was done to establish whether there is an optimum concentration of trehalose for preservation. The antibodies tested were mouse monclonal IgM anti A antibody, a mouse monoclonal IgM anti B antibody and a human monoclonal IgM anti Rh antibody used above. They were diluted to an appropriate starting concentration as follows:- mouse

anti A 1:10, mouse anti B 1:10, human anti Rh (to a final concentration of 20µg/ml). These were titrated 1:3 across a 96 well microtitre plate in Dulbecco's phosphate buffered saline (PBS) in a final volume of 50µl. To each horizontal 8 well row was added 50µl of trehalose at concentrations ranging from 10% w/v to 0.1% w/v. The wells were then dried at 37° overnight. Next day the wells were rehydrated with 100µl distilled water containing buffer sufficient to restore isotonicity. Then 50µl aliquots were transferred to a v-bottom microtitre plate and 25µl of 1% washed human red cells carrying the appropriate blood group antigens added and mixed. Haemagglutination titres were read after 2-3 hours at room temperature. The results showed that with the human antibody maximum preservation of activity was 100% and occurred at 2.5% final concentration of trehalose at the drying stage. There was a slight decrease in titre at 5% final trehalose concentration. With the anti Aantibody any trehalose concentration above 1% preserved maximal activity and with the anti B antibody any concentration above 0.5% preserved maximum activity.

Note

Many other antibodies, both monoclonal and serum antibodies of the IgM or IgG class have been preserved in this way with trehalose. Preservation of activity is

always 100% or close to it. The antibody can be either bound to the plastic surface of the well of the titre plate by hydrophobic and charge interaction or can be bound to nitrocellulose or nylon membrance by similar forces or can be free in solution. In all cases the activity is preserved. Where the antibody is free in solution its function is fully retained i.e. agglutinating anti blood group substance antibodies cause vigorous agglutination of the red cells are drying, showing that they retain their capacity for multivalent binding and rehydrating in solution.

Example 4

Protection of the antibody activity in whole serum by drying in the presence of trehalose

A sheep antiserum to rat IgG immunoglobulin (K237) was titrated in a 96 well flat bottom microtitre plate as 1:3 steps (25µl serum in 50µl PBS). 50µl of 5% w/v trehalose in D.W was then added and the plate dried at 37° in a warm room for 48 hr.

The wells were reconstituted with 100µl distilled water. 50µl of the contents of each well were transferred to v bottom microtitre plates, DA rat erythrocytes were washed x3 in saline and sensitized by incubation for 2 hr at RT with tissue culture

supernatant of the monoclonal antibody R3/13 HLK which is specific for the A^a class 1 transplantation antigen on DA erythrocytes. The red cells were then washed x3 in saline and resuspended at 1% v/v in PBS + 1% BSA. The sensitized rat erythrocytes were added. At the same time fresh K237 serum was titrated as above as a positive non-dried control.

The capacity for the dried and fresh serum to cause indirect agglutination of the red cells sensitized by the rat antibody R3/13 was then read.

Results

Row	Treatment	Titre (cups)	Titre (dilution)
A	Dried + Trehalose	7	1:8,748
B	" "	7	"
C	" "	7	"
D	" "	7	"
E	Fresh titration	7	"
F	" "	7	"
G	" "	7	"
H	" "	7	"
A	Dried (no Trehalose)	2	1:36
B	" "	1	1:12
C	" "	1	1:12
D	" "	2	1:36

Conclusions

Trehalose preserves 100% of the antibody activity of the whole serum. Drying in the absence of trehalose destroys >99% of the activity.

Example 5

A number of different matrices were examined for use as a trehalose support. They include pure cellulose paper (Whatman No. 1 filter paper), glass fibre paper (Whatman GF/A and GF/C papers), polyurethane sponge foam and porous polyethylene sheet. These matrices were soaked in trehalose dissolved in distilled water at various concentrations from 10% down to 1% and dried at 37°. It was found that the content of trehalose in the dried paper was about twice that in the solution. Thus a 10% solution gave dry paper containing 20% by weight of trehalose and a 5% solution gave paper containing 10% by weight of trehalose. Various sera were applied to the paper and dried at 37°. After various periods of storage at 37°, samples were eluted with distilled water or normal saline; usually used in a volume 2-4 times that of the original serum sample so that their potency could be directly compared with fresh non-dried samples of the same sera.

K237, a sheep antiserum against rat IgG, was dried on to 10% trehalose-impregnated cellulose paper, glass fibre paper and porous polyethylene sheet and stored for 7 days at 37°.

The paper or sheet was eluted with 4 volumes of normal saline and titrated in an indirect haemagglutination assay for its ability to agglutinate DA strain rat erythrocytes coated with a monoclonal antibody (JN2/85) against the class 1 antigens expressed on the erythrocyte membranes. The results show that 100% of the activity was preserved on cellulose paper and on porous polyethylene sheet, and 50% on glass fibre paper.

Paper	Titre
Schleicher & Schull GB003	1:640
Schleicher & Schull GB002SB	1:640
Whatman GF/C (Glass fibre)	1:1280
Whatman No.1 (Cellulose)	1:2560
Porous Polyethylene Sheet	1:2560
(non-dried antiserum	1:2560)

The same antiserum was then tested by drying on the Whatman No.1 cellulose paper in the presence of various sugars and in the absence of sugar.

Sugar	Titre
Sucrose	1:640
Raffinose	1:640
Trehalose	1:1280
Nil	1:640
Wet antiserum	1:1280

Note that a drop in tire from 1:1280 to 1:640 means at least 50% of antibody activity is lost. No significant difference exists between the sucrose and raffinose papers and the untreated paper.

Example 6

Drying phycoerythrin on paper or membranes

R-phycoerythrin at 5 mg/ml was dried at 37° on glass fibre filter paper (Whatman GF/C), cellulose filter paper (Whatman No. 1) or on nitrocellulose membrane (Schleicher & Schull 0.45μ pore size) in the presence of 5% trehalose in solution and in its absence. In the absence of trehalose the spots of dried R-phycoerythrin changed colour to a purplish red and lost >90% of their fluorescence intensity. In the presence of added trehalose they retained both colour and fluorescence. The trehalose-containing samples were stored in the dark for 10 months without loss of fluorescence.

Example 7

Use of membrane-dried phycoerythrin in a novel assay for probing DNA on nitrocellulose membranes.

Because fluorescence of phycoerythrin can be excited in the dry state on membranes without fading, a very strong integral fluorescent signal can be recorded on photographic emulsion from this molecule.

As an example of the sensitivity of this method, 10 fold serial dilutions of DNA from 100 picogram to 0.1 pg were dried onto nitrocellulose membranes using a slot-blot apparatus. This DNA had been labelled with biotin using photobiotin. These membranes were probed with streptavidin covalently coupled to R-phycoerythrin (QB-AR 4 Serotec Ltd.) by incubation for 1 hr at room temperature in a 1 mg/ml solution followed by extensive washing overnight in 300 ml of blocking buffer (1% BSA in PBS). The membranes were then washed in a solution of 10% trehalose in distilled water and dried at room temperature. The membranes were then photographed using a 1 hr exposure in a modified Polaroid VDU camera while illuminated with intense blue light from a xenon light source passed through 490 nm interference filters (Ealing Beck). The camera lens was covered with a long pass interference filter with a 560 nm cutoff so that

only the orange-red fluorescence was recorded. The blots of labelled DNA were readily detected with a sensitivity of > 10 pg DNA and negligible background. The same dried membrane of nitrocellulose was photographed with this system more than 20 times over a three day period without significant fading of the fluorescence signal. In contrast, when illuminated wet and in the absence of trehalose, the fluorescence signal fades within about 30 seconds. Drying in the absence of trehalose leads to loss of the fluorescent signal.

Example 8

Fluorescence microscopy of rat lymphocytes

Fluorescence microscopy using R-phycoerythrin as the fluorochrome was previously very inefficient because of fluorescence fading in aqueous mountants. This can be prevented by drying of the preparation and mounting in a non-polar mountant such as Gurr's Fluoromount, but drying destroys more than 90% of the fluorescence of R-phycoerythrin. This is prevented by drying in the presence of trehalose in the final buffer. This preserves virtually all of the fluorescence intensity of the labelled cells or tissues which can be mounted in an organic non-aqueous mountant and repeatedly examined for prolonged periods without loss of fluorescent signal.

The anti CD4 monoclonal antibody W3/25 was coupled with biotin-linker-arm N-hydroxysuccinimide ester and used in saturating doses to label the T helper subset of rat lymphocytes. These labelled cells were then detected with a second stage staining using a streptavidin-R-phycoerythrin conjugate. After a final wash the cells were divided into four aliquots and one was examined in the FACS to illustrate correct labelling with these reagents. Two other aliquots were spun down and resuspended in serum which either contained 10% added trehalose or did not. Smears were made and dried and then mounted in Fluoromount. The fourth aliquot was mounted wet in PBS. All three preparations were examined in a Zeiss photomicroscope II with Ploem optics.

The wet mount showed about 75% brilliantly fluorescent cells which faded rapidly so that within about 20-30 secs only the brightest cells could be barely discerned. The dried mount which did not contain trehalose showed only very weakly fluorescent cells which could barely be seen above the background.

The preparation dried with added trehalose showed 75% brilliantly fluorescent cells which did not fade. This preparation was repeatedly examined over several weeks and showed no detectable fading of the labelled cells.

Thus trehalose preserves completely several widely divergent and very labile properties of protein molecules even when they are completely dried. These properties (fluorescence and antigen binding) depend completely on the preservation of an intact tertiary structure of the molecules.

This phenomenon means that the functions and structures of proteins can now be completely preserved in the dry state. Thus proteins of scientific or medical interest can be preserved without the requirements for freezing or freeze-drying. Kits for immuno- assays can be prepared and stored dry without the need for sealing or liposome-formation to prevent water loss. Proteins and other molecules with novel and unusual properties such as chemiluminescence, conduction of electric currents and even possibly complex properties such as nitrogen fixation or photosynthesis may be preserved in the dry state.

Example 9

Preservation of enzyme activity

The mamalian enzyme alkaline phosphate from calf intestine was serially diluted in phosphate buffered saline (PBS) and added to the wells of a NUNC Immunoplate. After overnight incubation to bind the

enzyme to the plastic surface, the wells were washed, shaken free of liquid, and dried at 37° in the presence or absence of 5% trehalose in distilled water. The plates were then incubated at room temperature for 4 weeks. At the end of this time the wells were rehydrated with distilled water and substrate added. A positive control consisted of fresh dilutions of the enzyme. The activity of one enzyme was measured by the standard test of reducing p-nitrophenylphosphate and monitoring the optical density at 405 nm.

Full retention of activity was obtained when the enzyme was dried with trehalose, compared with over 90% loss of activity when it was dried in the absence of trehalose. Identical results were obtained with horseradish peroxidase when used in identical experiments.

Example 10

Preservation of Serum Complement

Guinea pig serum was prepared fresh and frozen in aliquots at -196° until use. As target cells, washed sheep erythrocytes (SRBC) which had been stored in Alsever's solution for up to 2 weeks were incubated with the bovine monoclonal anti-Forrsman antibody 94A1-AZA for 1 hr at 4° and then washed in complement fixation

diluent (CFD).

Freshly thawed guinea pig serum was diluted in CFD where required in flat bottom microtitre plates (NUNC, Denmark) and trehalose added at various concentrations. The plates were dried under a stream of dry air at room temperature overnight.

For the CH50 assay (P.J. Lachmann and M.J. Hobart in "Handbook of Experimental Immunology" ed. D.M. Weir, Blackwell 1978), dried or fresh complement was titrated out in a U bottom microtitre plate and sensitised SRBC added to a final concentration of 2%. The plates were then incubated at 37° for 1 hr and centrifuged. The endpoint was measured as the cup in which 50% of the erythrocytes remained as a button.

Results

The protective effect of trehalose depended upon the molar ratio of sugar to protein. Thus 100% of complement activity was preserved in 50% normal serum by trehalose at 0.22M and above, in 25% serum this required 0.15M and above. High concentrations of trehalose (up to 0.5M) were not inhibitory at any concentration of serum.

Drying of neat serum in the absence of trehalose

caused a loss of 75% of the complement activity. Drying of 25% serum in the absence of trehalose caused a loss of 93% of complement activity. Those loses were completely prevented by trehalose, the activity of dried and reconstituted complement being exactly equivalent to fresh complement.

Example 11

Agarose gels

We have dried 2 and 3% agarose gels containing 2-20% trehalose and compared their rehydration characteristics with gels dried without trehalose. The extent of rehydration was measured by weighing the gels to establish the amount of water regained. Their suitability as separation media was established by preparing Ouchterlony double diffusion assays and immunoelectrophoresis in the rehydrated, trehalose-preserved, dried gels.

The results (Table) show 100% water regain by 2% and 3% gels dried from 5% or 10% trehalose. These gels are identical in appearance with freshly poured and solidified gels.

Both Ouchterlony diffusion and immunoelectrophoresis can be performed in such gels with results which are

identical to assays performed in fresh gels. By varying the buffer in which the gels are reconstituted, the same gel, dried from agarose/trehalose in distilled water, can be used for a variety of purposes. Thus Ouchterlony buffer makes the gel suitable for this assay while electrophoresis buffer enables electrophoresis or immunoelectrophoresis to be performed.

TABLE

		Weight (g)			
% Trehalose		Fresh Gel	Dry Film	Rehydrated Gel	Recovery %
2% Agarose	5	1.721	0.170	1.733	100
	10	2.390	0.345	2.371	99
	0	2. 08	0.067	0.727	35
3% Agarose	5	2.126	0.220	2.170	102
	10	2.280	0.343	2.352	103
	0	1.984	0.079	0.717	36

This technique means that agarose gels can be prepared in advance, dried and stored at room temperature indefinitely and can be reconstituted within 1 hr by soaking in buffer and used immediately.

CLAIMS:

1. A method of protecting proteins and other macromolecules against denaturation during drying, comprising subjecting an aqueous system containing the protein or other macromolecule to drying above freezing point in the presence of trehalose.

2. A method according to claim 1, in which the aqueous system contains from 0.05 to 20% by weight of trehalose.

3. A method according to claim 1, in which the ratio of trehalose to protein or other macromolecule is at least 1.4:1 by weight.

4. A method according to claim 1, in which the protein or other macromolecule is an enzyme, serum, serum complement, an antibody or antigen (either free or coupled to a support), a fluorescent protein, a vaccine component or a polysaccharide.

5. A method according to claim 1, in which the system is dried at ambient temperature or above and at atmospheric pressure.

6. A dried product containing trehalose and a protein or other macromolecule in a weight ratio of at least

1.4:1 respectively.

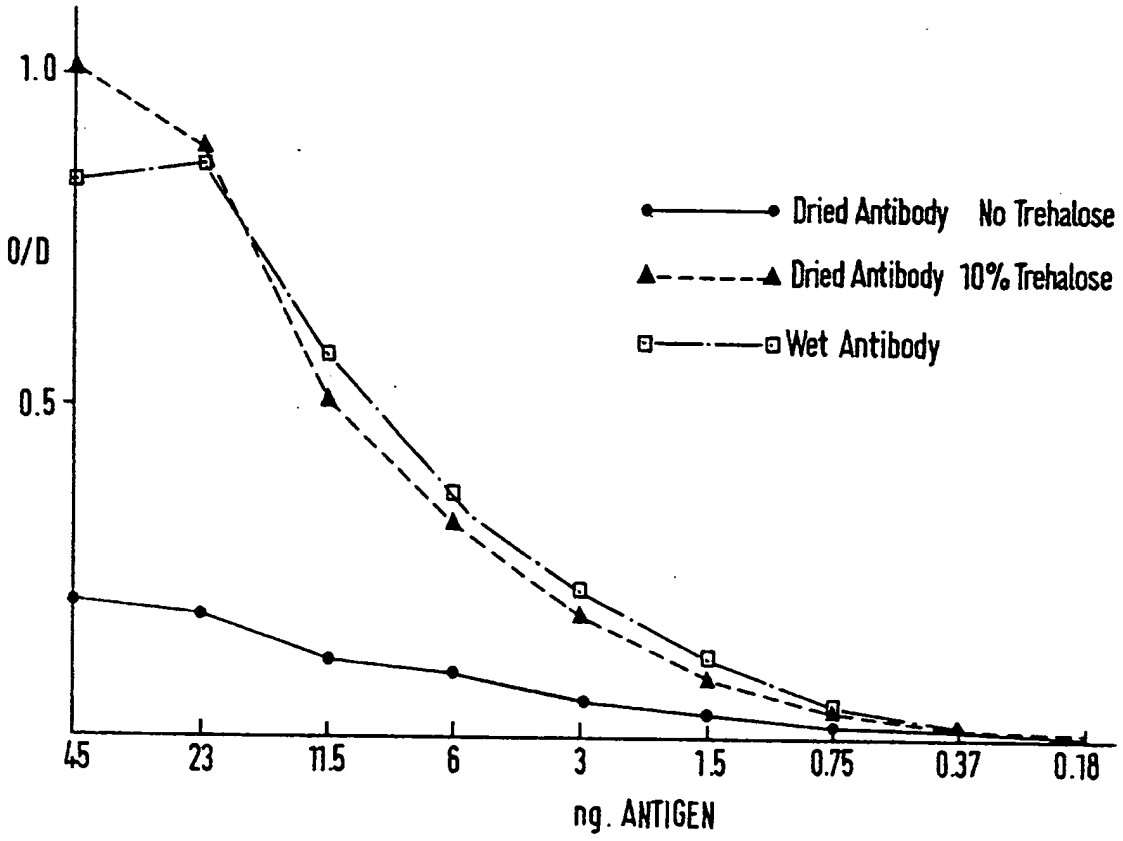
7. A dried product according to claim 6 containing an enzyme, serum complement, an antibody or antigen (either free or coupled to a support), a fluorescent protein, a vaccine component or a polysaccharide.

8. A plate having adhering thereto one or more antibodies dried in the presence of trehalose.

9. A porous matrix impregnated with trehalose, for use in a method according to claim 1.

10. A porous matrix according to claim 9, containing 5-25% by weight trehalose.

11. A porous matrix according to claim 9, comprising a cellulose or glass-based paper, a porous plastic film, or a fabric.



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 86/00396

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 9/96; G 01 N 33/531; G 01 N 33/543; G 01 N 33/52; A 61 K 39/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	G 01 N; C 12 N; C 12 Q; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X	Patents Abstracts of Japan, volume 9, no. 323 (414) (2046), & JP, A, 60149972 (YATORON K.K.) see the abstract	1,4,5,7
Y	EP, A, 0111216 (TAKEDA CHEMICAL INDUSTRIES, LTD.) 20 June 1984 see claim 4; page 20, lines 15-27	1-8
Y	US, A, 1855591 (L. WALLERSTEIN) 26 April 1932 see the whole document	1-8
Y	FR, A, 2406999 (BEHRINGWERKE AG) 25 May 1979 see page 2, lines 5-15; claims 1-5 & GB, A, 2009198 (cited in the application)	1-4,6,7
Y	FR, A, 2532178 (ASAHI KASEI KOGYO K.K. & DAINIPPON PHARMACEUTICAL CO., LTD.) 2 March 1984 see claims 1,12,13,16,20,21,22 & GB, A, 2126588 (cited in the application)	1,2,4,7

./.

* Special categories of cited documents: [14]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22nd October 1986	25 NOV 1986
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A, 0140489 (WAKO PURE CHEMICAL INDUSTRIES, LTD.) 8 May 1985 see page 7, lines 7-20; page 9, line 23 - page 10, line 12; claims 1-9 (cited in the application)	1,2,4,5,7,8
A		9-11
Y	GB, A, 2016687 (ABBOTT LABORATORIES) 26 September 1979 see the whole document	1,2,4,5,7,8
A		9-11

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 86/00396 (SA 13880)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 31/10/86

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0111216	20/06/84	EP-A,B	0037110	07/10/81
		JP-A-	56138248	28/10/81
		CA-A-	1162848	28/02/84
		US-A-	4517290	14/05/85
		JP-A-	57006363	13/01/82
		EP-A,B	0049898	21/04/82
		JP-A-	57118790	23/07/82
		CA-A-	1181342	22/01/85
		US-A-	4496658	29/01/85
US-A- 1855591		None		
FR-A- 2406999	25/05/79	BE-A-	871612	27/04/79
		GB-A,B	2009198	13/06/79
		DE-A-	2748132	03/05/79
		JP-A-	54070422	06/06/79
		US-A-	4206200	03/06/80
		CA-A-	1113861	08/12/81
		SE-A-	7811083	28/04/79
FR-A- 2532178	02/03/84	JP-A-	59039829	05/03/84
		GB-A,B	2126588	28/03/84
		DE-A,C	3331003	01/03/84
		US-A-	4457916	03/07/84
		JP-A-	59059625	05/04/84
EP-A- 0140489	08/05/85	JP-A-	60035263	23/02/85
GB-A- 2016687	26/09/79	NL-A-	7902152	24/09/79
		BE-A-	874956	19/09/79
		FR-A-	2420762	19/10/79
		DE-A,C	2910707	04/10/79
		JP-A-	54128396	04/10/79
		AU-A-	4419579	27/09/79
		CA-A-	1086647	30/09/80
		AU-B-	527489	10/03/83
		SE-A-	7902389	21/09/79

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 222 313**
A2

(12) # EUROPEAN PATENT APPLICATION

(21) Application number: 86115332.8

(22) Date of filing: 05.11.86

(51) Int.Cl.⁴: A 61 K 31/55
A 61 K 31/415, A 61 K 31/19-5

(30) Priority: 14.11.85 US 798032

(43) Date of publication of application:
20.05.87 Bulletin 87/21

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(71) Applicant: THE ROCKEFELLER UNIVERSITY
1230 York Avenue
New York, NY 10021(US)

(72) Inventor: Cerami, Anthony
Ram Island Drive
Shelter Island New York 11964(US)

(72) Inventor: Ulrich, Peter
500 East 63rd Street
New York New York 10021(US)

(72) Inventor: Brownlee, Michael
500 East 63rd Street
New York New York 10021(US)

(74) Representative: Denmark, James et al,
5 York Place
Leeds LS1 2SD Yorkshire(GB)

(54) **Method and agents for inhibiting protein aging.**

(57) The present invention relates to compositions and methods for inhibiting protein aging. Accordingly, a composition is disclosed which comprises an agent or compound capable of inhibiting the formation of advanced glycosylation end products of target proteins by reacting with the carbonyl moiety of the early glycosylation product of such target proteins formed by their initial glycosylation. Suitable agents may contain an active nitrogen-containing group, such as a hydrazine group, and may further be at least partially derived from amino acids. Particular agents comprise aminoguanidine, α-hydrazinohistidine and lysine. The method comprises contacting the target protein with the composition. Both industrial and therapeutic applications for the invention are envisioned, as food spoilage and animal protein aging can be treated.

EP 0 222 313 A2

This invention was made with partial assistance from grants from the National Institutes of Health and the Brookdale Foundation.

RELATED PUBLICATIONS

The Applicants are co-authors of the following articles directed to the subject matter of the present invention: "COVALENT ATTACHMENT OF OF SOLUBLE PROTEINS BY NONENZYMATICALLY GLYCOSYLATED COLLAGEN: ROLE IN THE IN SITU FORMATION OF IMMUNE COMPLEXES", Brownlee M., Pongor S., Cerami A., (1983), J. EXP. MED., Vol. 158, pp. 1739-1744; and "AGING OF PROTEINS: ISOLATION AND IDENTIFICATION OF A FLUORESCENT CHROMOPHORE FROM THE REACTION OF POLYPEPTIDES WITH GLUCOSE", Pongor, S., Ulrich, P., Bencsath, A.A., and Cerami, A., PROC. NATL. ACAD. SCI. USA, Vol. 81, pp. 2684-2688, (May, 1984), both of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates generally to the aging of proteins resulting from reaction of glucose, and particularly to the nonenzymatic glycosylation of proteins and subsequent reactions leading to advanced glycosylation end products, and to methods and agents for their inhibition.

The reaction between glucose and proteins has been known for some time. Its earliest manifestation was in the

appearance of brown pigments during the cooking of food, which was identified by Maillard in 1912, who observed that glucose or other reducing sugars react with amino acids to form adducts that undergo a series of dehydrations and rearrangements to form stable brown pigments. Maillard, L.C. (1912) C.R. Acad. Sci., Vol. 154, pp. 66-68.

In the years that followed the initial discovery by Maillard, food chemists studied the hypothesized reaction in detail and determined that stored and heat treated foods undergo nonenzymatic browning as a result of the reaction between glucose and the polypeptide chain, and that the proteins are resultingly crosslinked and correspondingly exhibit decreased bioavailability. Finot, P.A. (1982) in Modification of Proteins, eds, Feeney, R.E. and Whitaker, J.R., American Chemical Society, Vol. 198, pp. 91-124, Washington, D.C. At this point, it was determined that the pigments responsible for the development of the brown color that develops as a result of protein glycosylation possessed characteristic spectra and fluorescent properties, however the chemical structure of the pigments had not been specifically elucidated.

The reaction between reducing sugars and food proteins discussed above was found in recent years to have its parallel in vivo. Thus, the nonenzymatic reaction between glucose and the free amino groups on proteins to form a stable amino, 1-deoxy ketosyl adduct, known as the Amadori product, has been shown to occur with hemoglobin, wherein a rearrangement of the amino terminal of the β-chain of hemoglobin by reaction with glucose, forms the adduct known as hemoglobin A_{1c}. The reaction has also been found to occur with a variety of other body proteins, such as lens crystallins, collagen and nerve proteins. See, Bunn, H.F., Haney, D.N., Gabbay, K.H. and Gallop, P.H., (1975) Biochem. Biophys. Res. Comm. Vol.

67, pp. 103-109; Koenig, R.J., Blobstein, S.H. and Cerami, A.,(1977) J. Biol. Chem. Vol. 252, pp. 2992-2997; Monnier, V.M. and Cerami, A., (1983) in Maillard Reaction in Food and Nutrition, ed. Waller, G.A., American Chemical Society, Vol. 215, pp. 431-448; and Monnier, V.M. and Cerami, A., (1982) Clinics in Endocrinology and Metabolism Vol. 11, pp. 431-452. Moreover, brown pigments with spectral and fluorescent properties similar to those of late-stage Maillard products have also been observed in vivo in association with several long-lived proteins, such as lens proteins and collagen from aged individuals. An age related linear increase in pigment was observed in human dura collagen between the ages of 20 to 90 years. See, Monnier, V.M. and Cerami, A. (1981) Science, Vol. 211, pp. 491-493; Monnier, V.M. and Cerami, A., (1983) Biochem. Biophys. Acta, Vol. 760, pp. 97-103; and, Monnier, V.M., Kohn, R.R. and Cerami, A., "Accelerated Age-Related Browning of Human Collagen in Diabetes Mellitus", (1984) Proc. Nat. Acad. Sci. Vol. 81, pp. 583-587. Interestingly, the aging of collagen can be mimicked in vitro by the crosslinking induced by glucose; and the capture of other proteins and the formation of adducts by collagen, also noted, is theorized to occur by a crosslinking reaction, and is believed to account for the observed accumulation of albumin and antibodies in kidney basement membrane. See, Brownlee, M., Pongor, S. and Cerami, A., (1983) J. Exp. Med., Vol. 158, pp. 1739-1744; and Kohn, R.R., Cerami, A. and Monnier, V.M., (1984) Diabetes, Vol. 33, No. 1, pp. 57-59.

In parent application Serial No. 590,820, and in Pongor, S.M., et al., Supra., both incorporated herein by reference, a fluorescent chromophore was isolated and identified which was found to be present in certain browned polypeptides such as bovine serum albumin and poly-L-lysine, and was assigned the structure 2-furoyl-4(5)-2(furanyl)-1H-imidazole. The compound was found to exist in a tautomeric state and has incorporated

in its structure two peptide-derived amine nitrogens. The incorporation of these amine nitrogens and two glucose residues in the compound suggested that its peptide-bound precursor may be implicated in the in vivo crosslinking of proteins by glucose, which is observed in the late stage of the Maillard process. [See Chang, J.C.F., Ulrich, P.C., Bucala, R., and Cerami, A. (1985) J. Biol. Chem., Vol. 260, pp. 7970-7974]. This chromophore made possible the identification of the advanced glycosylation end products and assisted additional investigations seeking to clarify the protein aging process and if possible, to identify the specific chemistry involved in an effort to develop methods and agents for its inhibition. It is to this purpose that the present application is directed.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method and associated agents are disclosed for the inhibition of protein aging. In particular, agents for inhibiting protein aging due to the formation of advanced glycosylation end products may be selected from those materials capable of reacting with the early glycosylation product from the reaction of glucose with proteins and preventing further reactions. Thus, for example, compounds or compositions having active nitrogen-containing substituents such as hydrazine groups, have been theorized to be suitable, and compounds such as aminoguanidine, α- hydrazinohistidine and lysine have been found to be suitable. These agents appear to react with the early glycosylation product at its reactive carbonyl and thereby prevent the same from later forming the advanced glycosylation end products which lead to protein crosslinks.

The present invention also relates to a method for inhibiting protein aging by contacting the initially

glycosylated protein at the stage of the early glycosylation product with a quantity of one or more of the agents of the present invention. In the instance where the present method has industrial application, one or more of the agents may be applied to the proteins in question, either by introduction into a mixture of the same in the instance of a protein extract, or by application or introduction into foodstuffs containing the protein or proteins, all to prevent premature aging and spoilage of the particular foodstuffs.

In the instance where the present method has therapeutic application, the animal host intended for treatment may have administered to it a quantity of one of more of the agents, in a suitable pharmaceutical form. Administration may be accomplished by known techniques, such as oral, topical and parenteral techniques such as intradermal subcutaneous, intravenous or intraperitoneal injection, as well as by other conventional means. Administration of the agents may take place over an extended period of time at a dosage level of, for example, up to about 25 mg/kg.

The ability to inhibit the formation of advanced glycosylation end products carries with it significant implications in all applications where protein aging is a serious detriment. Thus, in the area of food technology, the retardation of food spoilage would confer an obvious economic and social benefit by making certain foods of marginal stability less perishable and therefore more available for consumers. Spoilage would be reduced as would the expense of inspection, removal and replacement, and the extended availability of the foods could aid in stabilizing their price in the marketplace. Similarly, in other industrial applications where the perishability of proteins is a problems, the admixture of the agents of the present invention in compositions containing such proteins would facilitate the extended useful life of the

same. Presently used food preservatives and discoloration preventatives such as sulfur dioxide, known to cause toxicity including allergy and asthma in animals, might be replaced with compounds such as those described herein.

The present method has particular therapeutic application as the Maillard process acutely affects several of the significant protein masses in the body, among them collagen, elastin, lens proteins, and the kidney glomerular basement membranes. These proteins deteriorate both with age (hence the application of the term "protein aging") and as one of the sequelae of diabetes. Consequently, the ability to either retard or substantially inhibit the formation of advanced glycosylation end products carries the promise of favorably treating a significant adverse effect of diabetes and of course, improving the quality and perhaps duration of animal life.

Accordingly, it is a principal object of the present invention to provide a method for inhibiting the extensive cross-linking of proteins that occurs as an ultimate consequence of the reaction of the proteins with glucose, by correspondingly inhibiting the formation of advanced glycosylation end products.

It is a further object of the present invention to provide a method as aforesaid which is characterized by a reaction with an initially glycosylated protein identified as early glycosylation products.

It is a further object of the present invention to provide a method as aforesaid which prevents the rearrangement and cross-linking of the said early glycoslyation products to form the said advanced glycosylation end products.

It is a yet further object of the present invention to provide agents capable of participating in the reaction with the said early glycosylation products in the method as aforesaid.

It is a still further object of the present invention to provide therapeutic methods for treating the adverse consequences of protein aging, manifest in the embrittlement of animal protein and the browning and spoilage of foodstuffs.

Other objects and advantages will become apparent to those skilled in the art from a consideration of the ensuing description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a graph showing the results of studies aimed at inhibiting the formation of advanced glycosylation end products in albumin which had been reacted with a quantity of glucose, on an in vitro basis.

FIGURE 2 is a graph showing the results of studies aimed at inhibiting protein entrapment and accumulation by glycosylated structural proteins such as collagen.

FIGURE 3A is a graph of the degree of solubilization of collagen incubated with glucose, with and without treatment with an agent of the present invention.

FIGURE 3B is a photograph of a polyacrylamide gel showing separation of protein fragments after cyanogen bromide digestion of collagen incubated with glucose with and without an agent of the present invention.

FIGURE 4 is a graph of the results of an in vivo study examining the extent of protein bound to the glomerular

basement membrane of diabetic rats to certain of which an agent of the present invention had been administered.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

In accordance with the present invention, a composition and associated methods have been developed which are believed to inhibit the formation of advanced glycosylation end products in a number of target proteins existing in both animals and plant material. In particular, the invention relates to a composition which may contain one or more agents that are capable of inhibiting the formation of advanced glycosylation end products on such target proteins, by reacting with the carbonyl moiety of the early glycosylation product that is formed by the initial glycosylation of the protein.

It is the carbonyl group located near the junction between the sugar and protein segments of the early glycosylation product that is theorized to comprise an active site that causes the further cross-linking of the protein to form the advanced glycosylation end product, and likewise contributes to the entrapment of other proteins that is evident in the development in vivo of conditions such as skin wrinkling, certain kidney diseases, atherosclerosis, osteoarthritis and the like. Similarly, plant material that undergoes nonenzymatic browning deteriorates and in the case of foodstuffs, becomes spoiled and inedible. Thus, the reaction with this carbonyl moiety is believed to inhibit the late stage Maillard effect.

The rationale of the invention is to use agents which block the post-glycosylation step, i.e., the formation of fluorescent chromophores such as that identified in Pongor, etal., supra. whose presence is associated with, and leads to, the adverse sequelae of diabetes and aging. An ideal agent would prevent the formation of the

chromophore and its associated cross-links of proteins to proteins and trapping of proteins on the other proteins, such as occurs in arteries and in the kidney.

The present invention does not attempt to prevent initial protein glycosylation, as it would be nearly impossible to use agents which prevent the reaction of glucose with protein amino groups. The agents that are capable of preventing initial glycosylation are likely to be highly toxic, and since the initial glycosylation comes to equilibrium in about three weeks, there is inadequate time available to achieve this objective. Instead, the ideal agent would prevent or inhibit the long-term, post-glycosylation steps that lead to the formation of the ultimate advanced glycosylation end product which are a direct cause of the pathology associated with aging and diabetes.

Accordingly, the compositions useful in the present invention comprise or contain agents capable of reacting with the active carbonyl intermediate of the early glycosylation product. Suitable agents include compounds having an active nitrogen-containing group or substituent such as a hydrazine group. Also, the agent or compound may be at least partially derived from an amino acid, including the esters and amides thereof, as compounds having this derivation are generally biocompatible with the target proteins to be contacted. For example, the agent may comprise a compound selected from the group consisting of aminoguanidine, α-hydrazinohistidine and lysine, and possibly mixtures of these agents or compounds. Each of these agents or compounds possesses an active nitrogen-containing substituent that is believed to react with the carbonyl of the early glycosylation product. Consequently, reaction of the agents with the glycosyl-lysine moiety of a protein would prevent this moiety from forming crosslinks with other groups.

Hollis and Strickberger (Diabetologia 28:282-5 [1985]) found that in vivo administration of the compound α-hydrazinohydrazine, a known inhibitor of the enzyme histidine decarboxylase, reduces the accumulation of albumin in the aortas of rats. The authors proposed that the drug acted to reduce production of histamine in this tissue, and that histamine is therefore the mediator of low density lipoprotein accumulation which is implicated in atherosclerotic disease. The findings of Hollis and Strickberger are distinguishable from the concept and application of the present invention on several grounds. A primary distinction is that the authors were concerned with protein accumulation that is observed in diabetic animals, and not advanced non-enzymatic glycosylation of proteins. Moreover, the mechanism of histamine synthesis supression by α-hydrazinohistidine suggested by the authors, is functionally distinct from the underlying concept of the present invention, and it is believed, may even be placed in question by the latter.

Thus, the agents of the present invention have been identified and tested on the basis of their ability to react with the carbonyl moiety of the early glycosylation product, and would not have been suggested from the work of Hollis and Strickberger. In particular, aminoguanidine is known to increase levels of histamine (See Lindberg, S., Tornqvist, A., "The Inhibitory Effect of Aminoguanidine on Histamine Catabolism in Human Pregnancy", ACTA OBSTET. GYNECOL. SCAND., 45: 131-139, 1966), and α-Hydrazinohistidine and aminoguanidine therefore have opposing effects on histamine levels. It can therefore be seen that the present findings that both α-hydrazinohistidine and aminoguanidine have efficacy in vivo and in vitro to reduce protein crosslinking rules out from consideration and consequently distinguishes the mechanism proposed by Hollis and Strickberger as the

explanation of the manner in which these drugs might work to reduce advanced glycosylation end product formation.

The compound aminoguanidine is known to have low toxicity in animals. According to the 1978 Registry of Toxic Effects of Chemical Substances, aminoguanidine base has a LD_{50} when administered subcutaneously of 1258 mg/kg in rats and 963 mg/kg in mice. The hydrochloride derivative had a LD_{50} in rats of 2984 mg/kg when given subcutaneously. The latter compound exhibits very low toxicity.

In the instance where the composition of the present invention is utilized for in vivo or therapeutic purposes, it may be noted that the compounds or agents used therein are biocompatible. Pharmaceutical compositions may be prepared with a pharmaceutically effective quantity of the agents or compounds of the present invention and may include a pharmaceutically acceptable carrier, selected from known materials utilized for this purpose. Such compositions may be prepared in a variety of forms, depending on the method of administration. For example, aminoguanidine may be derivatized to the hydrochloride salt from the commercially available bicarbonate salt to improve its solubility and to make it less irritating for intraperitoneal injection. Also, a liquid form would be utilized in the instance where administration is by intravenous or intraperitoneal injection, while, if appropriate, tablets, capsules, etc., may be prepared for oral administration. For application to the skin a lotion or ointment may be formulated with the agent in a suitable vehicle, perhaps including a carrier to aid in penetration into the skin. Other suitable forms for administration to other body tissues are contemplated.

The present invention likewise relates to methods for inhibiting the formation of advanced glycosylation end

products, which comprise contacting the target proteins with the composition of the present invention. In the instance where the target proteins are contained in foodstuffs, whether plant or animal origin, these foodstuffs could have applied to them by various conventional means a composition containing the present agents. Likewise, in the instance where therapeutic applications are intended, the animals to be treated would have administered to them a regular quantity of the pharmaceutical composition of the present invention. Administration could take place for example daily, and an effective quantity of the agent or compound of the present invention could range up to 25 mg/kg of body weight of the animal. A topical preparation may, for example, include up to 10% of the agent or composition in an ointment or lotion for application to the skin. Naturally, some variation in these amounts is possible, and the suggested amounts are provided in fulfillment of applicants' duty to disclose the best mode for the practice of the present invention.

As is apparent from a discussion of the environment of the present invention, the present methods and compositions hold the promise for arresting the aging of key proteins both in animals and plants, and concomitantly, conferring both economic and medical benefits as a result thereof. In the instance of foodstuffs, the administration of the present composition holds the promise for retarding food spoilage thereby making foodstuffs of increased self life and greater availability to consumers. Replacement of currently-used preservatives, such as sulfur dioxide known to cause allergies and asthma in humans, with non-toxic, biocompatible compounds is a further advantage of the present invention.

The therapeutic implications of the present invention relate to the arrest of the aging process which has as

indicated earlier, been identified in the aging of key proteins by advanced glycosylation and cross-linking. Thus, body proteins such as collagen, elastin, lens proteins, nerve proteins and kidney glomerular basement membranes would all benefit in their longevity and operation from the practice of the present invention. It is further theorized that the present invention would reduce the incidence of pathologies involving the entrapment of proteins by cross-linked target proteins, such as atherosclerosis, osteoarthritis, periarticular rigidity, loss of elasticity and wrinkling of skin, stiffening of joints, glomerulonephritis, etc. Likewise, all of these conditions are in evidence in patients afflicted with diabetes mellitus. Thus, the present therapeutic method is relevant to treatment of the noted conditions in patients either of advanced age or those suffering from one of the mentioned pathologies.

The present invention will be better understood from a consideration of the following illustrative examples, reviewing the selection and testing of certain of the agents of the present invention on both an in vitro and in vivo basis.

EXAMPLE I

To measure the ability of certain of the agents of the present invention to inhibit the production of advanced glycosylated end products in vitro, albumin and glucose were incubated together for two weeks in the presence of several test agents. Sample were taken at regular intervals for analysis. Advanced glycosylation endproducts were measured as appearance of fluorescent compounds, and early glycosylation products were measured by incorporation of radiolabeled glucose into albumin.

Reaction conditions were as follows. Each mixture contained 6 mg/mL bovine serum albumin, 200 mM glucose, 200 mM test agent (either aminoguanidine hydrochloride, α hydrazinohistidine, or lysine), and approximately 9.5 x 10^6 counts per minute of 14C-glucose in 0.5M phosphate buffer, pH 7.6. The radiolabeled glucose was prepurified before use to eliminate breakdown products which might react with the albumin and lead to an erroneous indication of the degree of early glycosylation product formation. Reaction mixtures were incubated at 37°C and samples were taken after 0.5, 1.0, 1.5, and 2 weeks. Control mixtures lacked glucose or agent.

After the incubation periods, samples were treated as follows. After dialysis to remove all unbound glucose, the amount of protein present was measured with a standard dye-binding assay. The amount of glucose which became bound to the albumin, a measure of early glycosylation products, was determined by precipitating albumin with trichloroacetic acid and measuring the radioactivity of the bound glucose using scintillation counting. Advanced glycosylation endproducts were quantitated by determining the fluorescence of the albuminas described in Parent Application Serial No. 590,820, and as described by Pongor et al., supra. Spectral measurements on excitation and emission maxima were made on all samples to ensure that these values had not been shifted as a result of adduct formation with inhibitors.

The results of this experiment are expressed graphically in FIGURE 1. For each sample, incorporation of radiolabeled glucose is indicated by the solid portion of the bar, and fluorescence is indicated in the open portion of the bar. All values are expressed as per milligram of albumin. In all further discussions, aminoguanidine refers to the hydrochloride derivative.

The results show that glucose and albumin react to form a large amount of fluorescent advanced glycosylation endproducts after 0.5, 1, 1.5, and 2 weeks of incubation ("GLUCOSE + BSA"). Inclusion of 200 mM aminoguanidine dramatically reduced by as much as eight-fold the formation of fluorescent compounds, by comparison with the control samples after a two week incubation ("BSA + GLUCOSE + I#2). Inclusion of 200 mM α-hydrazinohistidine also reduced formation of advanced glycosylation endproducts as measured by fluorescence ("BSA + GLUCOSE + I#1). Lysine appeared to cause an increase in fluorescent compound formation ("BSA + GLUCOSE + LYS"), but as will be seen in the next experiment, it had the ability to reduce protein crosslinking. The amount of early glycosylation endproducts, as measured by glucose incorporation, was nearly unchanged in all reactions. The control incubation without glucose showed little development of fluorescent products (A).

These results show that aminoguanidine, and to a lesser extent, α-hydrazinohistidine, reduce the formation of fluorescent compounds when glucose and albumin react over time, and indicate that these two agents reduce the amount of advanced glycosylation endproducts which form. The agents do not alter the formation of early glycosylation products.

EXAMPLE II

To more precisely measure the effect of the agents on the inhibition of protein crosslinking, an assay system was devised to measure the extent of in vitro binding of a soluble protein to an insoluble protein. This assay system mimicks the events which occur in tissues in which serum proteins become bound to proteins in extravascular matrix and which lead to accumulation of protein and narrowing of vessel lumina in several other tissues. These events in vivo give rise to kidney disease and

atherosclerotic disease, and lead to the pathologies associated with diabetes and aging.

To measure protein trapping (i.e., binding or accumulation), gelatin (collagen) was coupled to activated agarose beads (Affigel 10, Bio-Rad Laboratories) by routine methods. After coupling, all remaining active sites on the beads were quenched by reaction with glycine ethyl ester.

The beads were incubated for two weeks with bovine serum albumin and 400 mM glucose-6-phosphate, a more reactive form of glucose which forms early glycosylation products with proteins more rapidly than does glucose. Also included in some experiments were the test agents, aminoguanidine, α-hydrazinohistidine, or lysine, at a concentration of 200 mM. The bovine serum albumin was radioiodinated so that the amount which became bound to the beads could be measured. The amount of radiolabel that became bound to the beads in a direct measure of protein trapping.

After a two-week incubation of the reaction mixtures at 37°C, the beads were washed extensively with chaotropic agents and the covalently bound radioactivity was determined. The results are set forth in FIGURE 2.

The left bar shows the control level of radiolabel incorporated into the beads in the absence of glucose-6-phosphate and in the absence of any test agents ("CONTROL COLLAGEN"). The second bar shows the high amount of incorporation in the presence of glucose-6-phosphate ("NEG. COLLAGEN"). This is likened to the presence of high concentrations of glucose in the blood of uncontrolled patients with diabetes and the pathological sequelae which result.

The figure shows that the amount of protein trapping in the presence of either aminoguanidine ("NEG. COLLAGEN + I#2") or α-hydrazinohistidine ("NEG. COLLAGEN + I#1") is greatly reduced. Lysine alse reduced the amount of protein trapping to an extent similar to that of aminoguanidine (not shown). The results show the potential value of these compounds in vivo for reducing the trapping or soluble protein on to membranes and other tissues, and further evidence that these agents may be of value in reducing the pathogenesis of diabetes and aging.

EXAMPLE III

As a further evaluation of the compound aminoguanidine as a model for the prevention of protein trapping, crosslinking and formation of advanced glycosylation endproducts, the following experiment using calf skin collagen was performed. Collagen is a protein in the skin responsible for the suppleness of skin, and crosslinking leads to wrinkling, decreased elasticity, reduced susceptibility to proteolytic degradation, and other changes.

Collagen from samples of calf skin were extracted into acetic acid and then precipitated with 0.6 M sodium chloride. These procedures removed from the solution skin collagen that was already permanently crosslinked or denatured. Native collagen fibrils were reformed by dialysis against 0.02M phophate buffer and these were incubated for 3 weeks at 35°C in the presence of 140 mM glucose and with or without 200 mM aminoguanidine. After incubation, the samples were dialyzed and the degree of crosslinking was determined by two methods. First, the amount of reacted collagen which could be solubilized by treatment in 2% sodium dodecyl sulfate at 100°C was measured.

As shown in FIGURE 3A, collagen incubated with glucose and aminoguanidine was as soluble as collagen incubated in buffer alone. In contrast, collagen incubated in glucose without aminoguanidine was only 50% as soluble. This is further evidence that aminoguanidine may have utility in the prevention of age-related changes in skin and other tissues.

The reacted collagen was further examined by cleaving it into fragments using cyanogen bromide treatment in formic acid. The resulting protein fragments were separated by size by sodium dodecyl sulfate - polyacrylamide gel electrophoresis. After electrophoresis, the protein fragments were identified in the gel using silver staining. The gel is shown in FIGURE 3B.

Lane B contains collagen that was incubated with glucose alone. It is noted that a large amount of high molecular weight fragments form a continuous band at the top of the gel, indicating a large range of high molecular weight fragments. Some of this material could not enter the gel and is present in the 30% stacking gel above the gradient gel. Lane C contains the collagen incubated with glucose and aminoguanidine, and it is noted that there is no large amount of high molecular weight material at the top of the lane, as all of the protein fragments separate well in the lower part of the gel. Lane A shows collagen incubated in PBS alone. The far left lane is a series of molecular weight markers. Identical results were observed with and without the presence of disulfide bond reducing agents in the electrophoresis buffer.

The above data indicate that aminoguanidine reduces the amount of crosslinking which occurs when collagen is incubated with glucose, and suggest the utility of this agent for topical application to skin to prevent

age-related changes, including loss of elasticity and wrinkling.

The above in vitro experiments all point to the value of aminoguanidine as an agent to inhibit the formation of advanced glycosylation endproducts which form in vitro from proteins incubated in the presence of glucose. As glucose is present in the body and is elevated in amount in diabetes, and as proteins in the body are known to undergo crosslinking and form fluorescent compounds all indicative of advanced glycosylation endproducts, use of this agent in vivo might be useful in the prevention of the pathology associated with diabetes and changes that occur during aging.

Accordingly, the following experiment was performed to test the hypothesis of the present invention in an in vivo environment.

EXAMPLE IV

To measure the level of advanced glycosylation endproducts in vivo, the kidneys of rats were examined for serum proteins which became attached to glomerular basement membranes. This was determined to be a good model in which to study this process as it is known that significant kidney pathology occurs in untreated diabetes as a result of the build-up of extravasated plasma protein in the extravascular matrix in this organ.

The experiment consisted of giving both normal and diabetic rats daily intraperiteoneal doses of the agent aminoguanidine hydrochloride at a dose of 25 mg per kilogram of body weight, for a period of 16 weeks. The hydrochloride salt of aminoguanidine was used as it is more soluble and less irritating than the parent free base compound. Diabetes was induced prior to drug therapy with a single dose of streptozotocin. Control animals, both diabetic and normal, received no drug.

At the end of the agent therapy, animals were sacrificed and the kidneys were removed. Each organ was removed from its capsule and the medulla was removed. The remainder of the tissue, principally containing glomeruli, was frozen on dry ice and stored at -70°C. Tissue from 5 animals in each treatment group was combined for processing.

To prepare glomerular basement membranes, tissue was cut into slices and passed through a series of sieves (170, 100 and 270) to separate glomeruli from tubules and other undesired tissue elements as described (Beisswenger, P.J., Spiro, R.G., DIABETES, 22:180-193, 1973.). Glomerular purity was found to be 80-90%. The final material was collected and centrifuged at 1500 rpm for fifteen minutes to pellet the glomeruli, which were frozen at -70°C.

Thawed isolated glomeruli were then disrupted by treatment in a Branson Sonifier 200 cell disrupter for four one-minute intervals on ice with a one-minute rest between sonications. Samples were examined in a phase contrast microscope to ensure that all of the glomeruli were disrupted. Glomerular basement membranes were then pelleted by centrifugation at 3000 rpm for ten minutes, washed with 1 M sodium chloride followed by distilled water. The remaining pellet of purified glomerular basement membranes was frozen and lyophilized.

An enzyme immunoassay was used to measure the amount of serum immunoglobulin G (IgG) that became bound to the glomerular basement membranes of the normal and diabetic animals after treatment with and without the agent. To measure IgG, 6 mg samples of lyophilized glomerular basement membrane tissue was suspended in 0.5 mM of 0.05 M carbonate buffer, pH 7.6, and 0.5 mM of a 1:5,000 dilution of rat anti-IgG antibody conjugated to alkaline

phosphatase (Dynatech Corp.) was added. The mixture was incubated overnight in polystyrene tubes which were preblocked by incubation for two hours in 3% goat serum plus 0.05% Tween 20 in phosphate buffered saline (PBS), followed by two rinses in PBS plus Tween.

After overnight incubation to allow the antibody to bind to any IgG crosslinked to the glomerular basement membranes, the membranes were pelleted by centrifugation at 3200 rpm for five minutes and were washed free of the unbound antibody-enzyme conjugate with four rinses with PBS plus Tween followed by three rinses with distilled water. To measure the amount of antibody-enzyme conjugate remaining bound, 0.5 mM of substrate solution (containing 1 mg/mL para-nitrophenylphosphate in 10% diethanolamine, pH 9.8), was added and incubations were carried out for thirty minutes at room temperature. The reaction was stopped with the addition of 0.2 mL of M sodium hydroxide, and the absorbance at 400 nm was measured.

FIGURE 4 shows the results of this experiment. As can be seen, diabetic animals had a high level of IgG bound to their glomerular basement membranes ("D"), and normal animals had about one-fifth the amount ("N"). Diabetic animals which received daily doses of aminoguanidine hydrochloride showed the same low level of IgG in normal animals ("D + I"). Normal animals receiving the drug had about the same low level ("N + I").

These experiments indicated that aminoguanidine prevented the trapping and accumulation of this plasma protein on the rat glomerular basement membranes. Presumably the trapping of this and other serum proteins in the kidney, eye, on artery walls, and in other tissues known to be affected from this pathology would likewise be reduced. Trapping of lipoproteins on artery walls is known to contribute to atherosclerotic disease.

These in vivo experiments provide further evidence from the in vitro experiments that this type of drug therapy has benefit in reducing the pathology associated with the advanced glycosylation of proteins and the formation of crosslinks between proteins and other macromolecules. Drug therapy may be used to prevent the increased trapping and crosslinking of proteins that occurs in diabetes and aging which leads to sequelae such as arterial disease, including renal disease, hypertension, retinal damage, and extra-vascularly, damage to tendons, ligaments, and other joints. This therapy might retard atherosclerosis and connective tissue changes that occur with diabetes and aging. Both topical, oral, and parenteral routes of administration to provide therapy locally and systemically are contemplated.

This invention may be emobodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

WHAT IS CLAIMED IS:

1. A composition for inhibiting the advanced glycosylation of a target protein comprising an agent capable of reacting with the carbonyl moiety of the early glycosylation product formed by the initial glycosylation of the target protein.

2. A composition of Claim 1 wherein said agent comprises a compound having an active nitrogen-containing substituent.

3. A composition of Claim 2 wherein said active nitrogen-containing substituent is a hydrazine group.

4. The composition of Claim 2 wherein said compound is at least partially derived from a material selected from the group consisting of amino acids, their esters and amides, and mixtures.

5. The agent of Claim 2 wherein said compound is selected from the group consisting of aminoguanidine, α-hydrazinohistidine, lysine, and mixtures thereof.

6. A pharmaceutical composition for administration to an animal to inhibit the advanced glycosylation of a target protein within said animal, comprising a pharmaceutically effective amount of an agent capable of reacting with the carbonyl moiety of the early glycosylation product formed by the initial glycosylation of said target protein, and a pharmaceutically acceptable carrier.

7. The pharmaceutical composition of Claim 6 wherein said agent comprises a compound having an active nitrogen-containing substituent.

8. The pharmaceutical composition of Claim 7 wherein said active nitrogen-containing substituent is a hydrazine group.

9. The pharmaceutical composition of Claim 7 wherein compound is at least partially derived from a material selected from the group consisting of amino acids, their esters and amides, and mixtures.

10. The pharmaceutical composition of Claim 7 wherein said compound is selected from the group consisting of aminoguanidine, α-hydrazinohistidine, lysine, and mixtures thereof.

11. A method for inhibiting the advanced glycosylation of a target protein comprising contacting the target protein with an effective amount of a composition comprising an agent capable of reacting with the carbonyl moiety of the early glycosylation product formed by the initial glycosylation of the target protein.

12. The method of Claim 11, wherein said agent comprises a compound having an active nitrogen-containing substituent.

13. The method of Claim 12, wherein said active nitrogen-containing substituent is a hydrazine group.

14. The method of Claim 11, wherein said compound is at least partially derived from a material selected from the group consisting of amino acids, their esters and amides, and mixtures.

15. The method of Claim 11, wherein said compound is selected from the group consisting of aminoguanidine, α-hydrazinohistidine, lysine, and mixtures thereof.

16. The method of Claim 11, wherein said composition introduced into an isolated quantity of said target protein.

17. The method of Claim 11 wherein said target protein is found in foodstuffs and said composition is applied thereto.

18. A method for treating an animal to inhibit the formation of advanced glycosylation end products of a target protein within said animal, said method comprising administering an effective amount of a pharmaceutical composition, said pharmaceutical composition comprising an agent capable of reacting with the carbonyl moiety of the early glycosylation product formed by the initial glycosylation of said target protein.

19. The method of Claim 18 wherein said target protein is selected from the group consisting of collagen, elastin lens protein, blood vessel walls, nerve protein and glomerular basement membrane.

20. The method of Claim 18 wherein said pharmaceutical composition comprises said agent and a pharmaceutically acceptable carrier.

21. The method Claim 20, wherein said agent comprises a compound having an active nitrogen-containing substituent.

22. The method of Claim 21, wherein said active nitrogen-containing substituent is a hydrazine group.

23. The method of Claim 21, wherein said compound is at least partially derived from a material selected from the group consisting of amino acids, their esters and amides, and mixtures.

24. The method of Claim 21, wherein said compound is selected from the group consisting of aminoguanidine, α-hydrazinohistidine, lysine, and mixtures thereof.

25. The method of Claim 18, wherein said pharmaceutical composition is administered parenterally.

26. The method of Claim 18, wherein said pharmaceutical composition is administered topically.

27. The method of Claim 18, wherein said pharmaceutical composition is administered orally.

28. The method of Claim 18 wherein said pharmaceutical composition is administered regularly and daily.

29. The method of Claim 18 wherein said pharmaceutical composition is administered in an amount of up to about 25 mg/kg body weight of said animal.

30. The method of Claim 26 wherein said pharmaceutical composition is prepared in an ointment form and said agent is present in an amount of up to about 10% by weight.

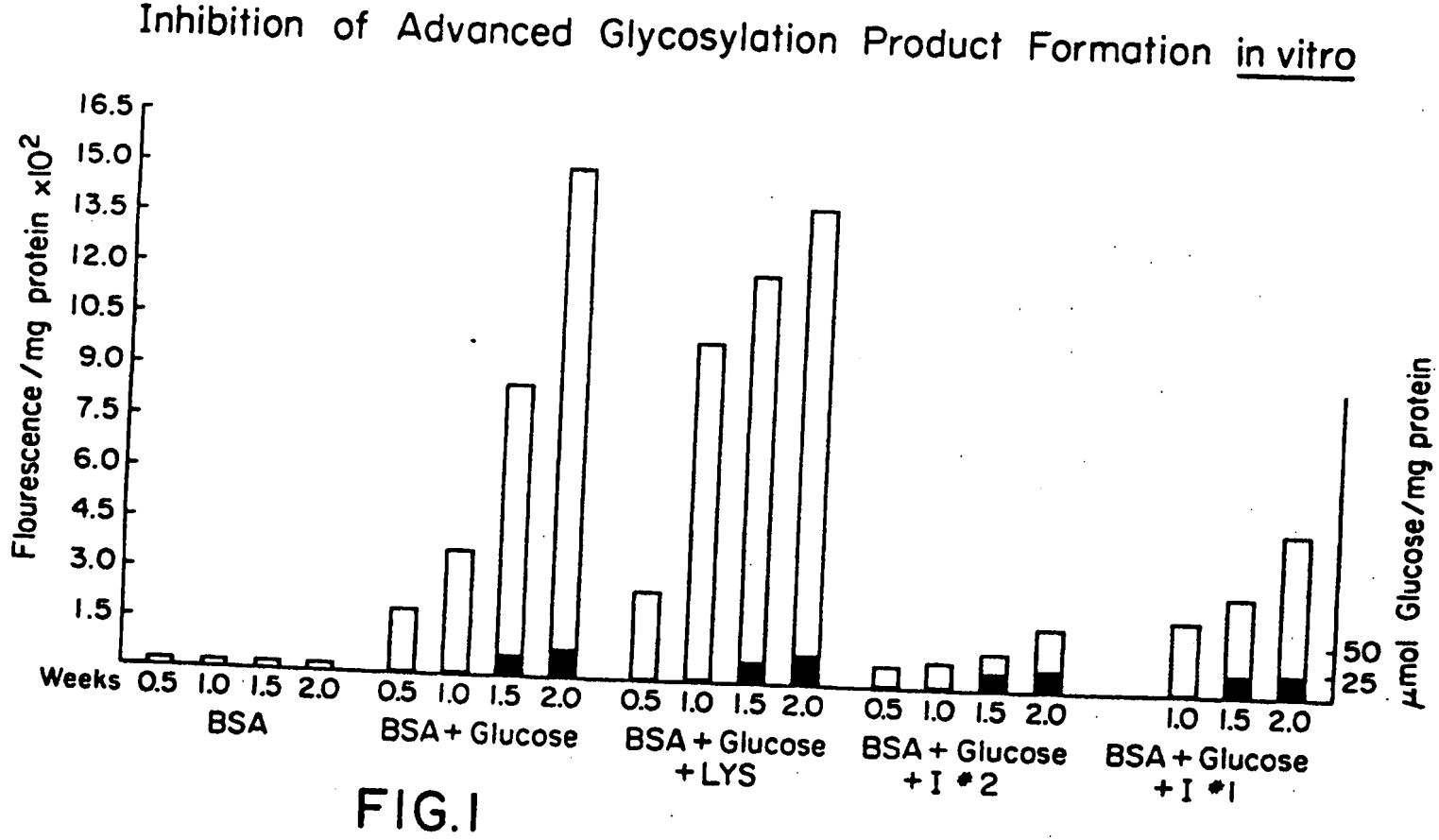


FIG.1

0222313

FIG.2

Covalent Trapping of BSA by Nonenzymatically Glycosylated Collagen

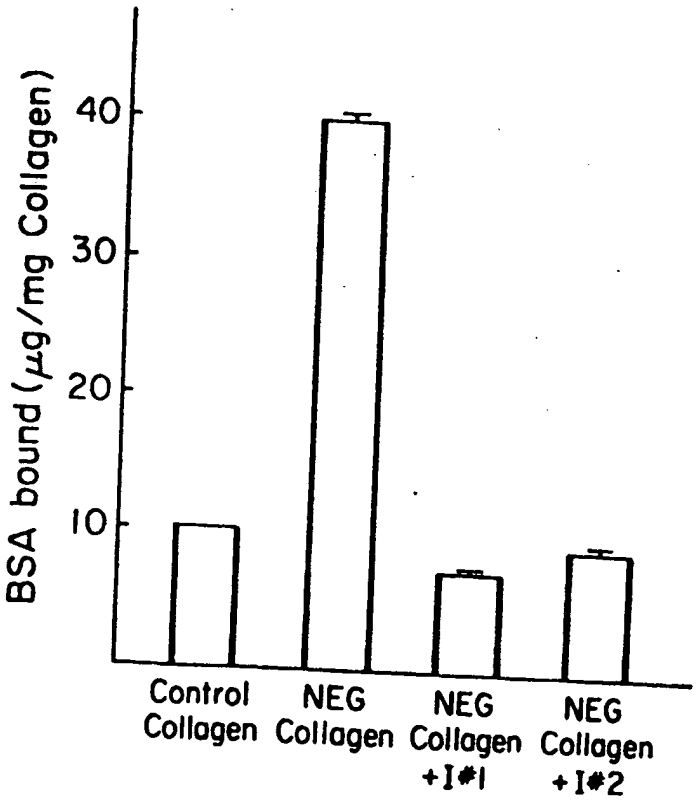
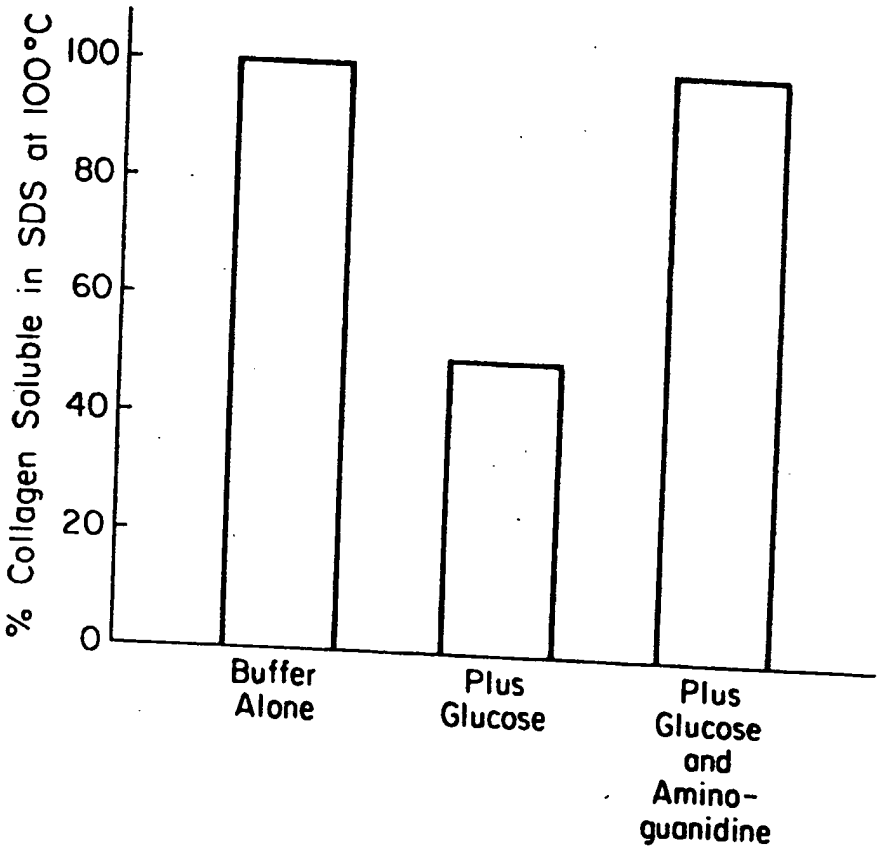


FIG.3A


% Collagen Soluble in SDS at 100°C
100
80
60
40
20
0
Buffer Alone
Plus Glucose
Plus Glucose and Amino-guanidine

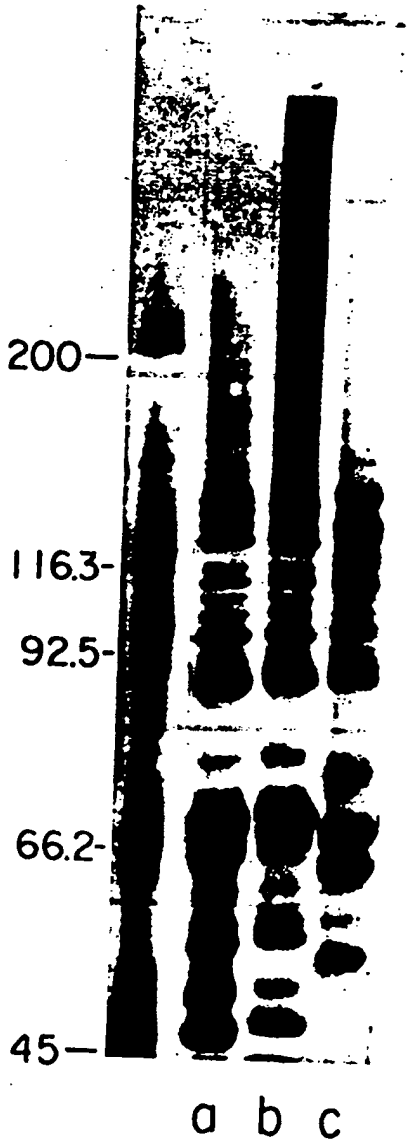


FIG.3B

FIG.4

Inhibition of Advanced Glycosylation Product Formation in vivo